Notch Signaling Drives Stemness and Tumorigenicity of Esophageal Adenocarcinoma

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ABSTRACT

Esophageal adenocarcinoma (EAC) ranks sixth in cancer mortality in the world and its incidence has risen dramatically in the western population over the last decades. Data presented herein strongly suggest that Notch signaling is critical for EAC and underlies resistance to chemotherapy. We present evidence that Notch signaling drives a cancer stem cell phenotype by regulating genes that establish stemness. Using patient derived xenograft models we demonstrate that inhibition of Notch by gamma-secretase inhibitors (GSI) is efficacious in downsizing tumor growth. Moreover, we demonstrate that Notch activity in a patient’s EUS-derived biopsy might predict outcome to chemotherapy. Therefore, this study provides a proof of concept that inhibition of Notch activity will have efficacy in treating EAC, offering a rationale to lay the foundation for a clinical trial to evaluate the efficacy of GSI in EAC treatment.
INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer death in the world (1). The incidence of esophageal adenocarcinoma (EAC), a subtype of esophageal cancer, has been on the rise in the United States and other western countries over the past 30 years (2–4). The prognosis for esophageal adenocarcinoma is poor, with a 5-year survival rate of 19% (5) and only 0.9% for advanced EAC (6). Currently, a detailed molecular understanding of the underlying pathophysiology of EAC has not been realized. However, it is generally accepted that the etiological condition that drives the onset of EAC is gastrointestinal reflux disease followed by a metaplastic condition termed Barrett’s Esophagus (BE) (7,8). Although the link is compelling, only a small percentage of Barrett’s patients progress to frank adenocarcinoma (9). Similarly, the molecular details that drive progression from BE to EAC remain poorly understood. Therefore it is imperative that a detailed knowledge of the molecular mechanisms driving EAC is obtained in order to develop more effective treatment strategies and improve clinical management of EAC.

Given that Notch acts as an oncogene and is aberrantly re-activated in many human neoplasms, we sought to determine a role for Notch in EAC (10,11). Herein, we present evidence that Notch signaling is greater in the less differentiated tumors and drives a cancer stem cell phenotype. We demonstrate that Notch signaling is critical for these EAC CSCs and regulates genes that establish stemness. Using patient derived xenograft models we demonstrate that inhibition of Notch signaling by gamma-secretase inhibitors (GSI) is efficacious in downsizing tumor growth. Moreover, we provide evidence that demonstrates
Notch activity in a patient’s EUS-derived biopsy sample can predict outcome to chemotherapy. Therefore, it appears that Notch signaling is driving resistance to chemotherapy by maintaining a robust population of CSC and that inhibition of Notch depletes the CSC population and sensitizes cells to chemotherapeutic agents which should lead to a better and more durable response to neoadjuvant chemotherapy (NAC).
MATERIALS AND METHODS

Human Esophageal Adenocarcinoma and Normal Esophageal Mucosa Samples

Human EAC tumors, matched adjacent non-tumor tissues, and normal esophageal mucosa were obtained from tissue microarray (Biomax.US, ES8011) and the patients undergoing surgery at Miller School of Medicine, University of Miami. We obtained consent from all patients and approval from the Institutional Research Ethics Committee.

Cell Culture

Human EAC cell lines OE33 and OE19 were obtained from the European Collection of Cell Culture. FLO1 and JH-EsoAd1 cells were a generous gift from other labs (see acknowledgements). Normal human primary esophageal epithelial cells EAC09N, EAC10N, and EAC11N were isolated from human esophageal mucosa obtained from normal adjacent tissue. Het-1A, a human immortalized esophageal epithelial cell line, was obtained from ATCC. All cell lines were characterized by short tandem repeat analyses (STR) profiling (LGS Standards SLU, Barcelona, Spain) fewer six months after receipt.

Immunohistochemistry and Immunofluorescence

Immunohistochemical staining of NICD (1:200; ab-8925) and DLL4 (1:200; ab-7280) were carried out using a DAKO autostainer. Anti-rabbit IgG labeled with red-fluorescent Alexa Fluor 594 dye (1:200; Invitrogen A21207) was used as the secondary antibody for immunofluorescence.

Quantitative RT-PCR

Total RNA was isolated and cDNA was synthesized according to the manufacturer’s protocol.
Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermal cycler conditions were 50°C for 2 minutes and 95°C for 10 minutes, then 40 cycles of 15 seconds at 95°C (denaturation) followed by 1 minute at 59°C (annealing and extension) (12). GAPDH was used to normalize gene expression. All samples were normalized to the relative levels of GAPDH and results expressed as fold increase in relative levels of all.

**Western Blotting**

Cells lysates were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in milk and incubated with the antibodies followed by incubation with the anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase. For detection, enhanced chemiluminescence reaction (Amersham Biosciences) was done according to the manufacturer’s specification.

**Lentiviruses and Infection**

Lentiviruses expressing various shRNAs and over-expression plasmids were produced as described previously (13). For viral infection, sub-confluent cells were overlaid with the virus-containing medium and fresh growth medium in the presence of polybrene (Sigma).

**Luciferase Assay**

Cells grown in 24-well plates were transiently transfected with CSL/GFP reporter plasmid using Lipofectamine 2000 (11668-019; Invitrogen) and luciferase activity was measured in cell lysates after 24 hours.

**Colonies Formation Assay and Cell Viability Assays**

Cells were cultured at low density under treatment, and then colonies were stained with 0.01%
crystal violet and counted. The cells were measured using the Cell Titer-Glo assay (G7572; Promega) for Cell Viability Assays.

**Tumor Sphere Formation Assay**

To obtain tumor spheres, cells were cultured in DMEM/F12 with 2% B-27 serum-free supplement (17504-044; Invitrogen), 20 ng/ml epidermal growth factor (EGF; PHG0311L; Invitrogen), and 20 ng/ml basic fibroblastic growth factor (FGF; PHG0266; Invitrogen) for 14 days to select for CSCs and early progenitor cells. Resulting tumor spheres were examined and counted under the microscope.

**Flow Cytometric Analysis of Aldehyde Dehydrogenase (ALDH)**

Cells were stained using ALDEFLUOR kit (Stem Cell Tech) following the manufacturer’s instructions and were analyzed by flow cytometry, as described previously (14).

**Chromatin Immunoprecipitation (ChIP) Assay**

OE33 and FLO1 cells were cross-linked with 1% formaldehyde and cross-linking was quenched by adding glycine to a final concentration of 0.125 M. Cells were resuspended in SDS lysis buffer and sonicated to yield chromatin fragments of approximately 300 to 800 bp. Lysates were immunoprecipitated with α-Notch 927 (polyclonal), α-Notch (ab27526, Abcam), or α-Pragmin (Bethyl Laboratories, Montgomery, TX) antibodies and were reverse cross-linked at 65°C in 200 mM NaCl for 4 h followed by incubation with RNase A and proteinase K. DNA was cleaned using PCR purification kit (Qiagen) and Hes1 and GAPDH were amplified by qPCR. Primer sequences are available upon request.

**Animal Experiments**
Six-week-old SCID/hairless mice and CD-1 Nude mice were purchased from Charles River Laboratories, and NOD-SCID gamma (NSG) mice from Jackson Laboratories. Animal experiments were approved by the University of Miami Institutional Animal Care and Use Committee. EAC cells were injected subcutaneously. When the tumor size reached 200mm$^3$, the mice were split into two groups uniformly. PDX cancer models were established as described previously (15) in NSG mice. Tumor volume was measured by the formula: Volume = (S×S×L)/2 (15). The xenografts were harvested and samples were subjected to histological examination.

**Genome-Wide Expression Meta-Analysis**

The genome wide expression data from 64 EAC patients using Illumina human-6 v2.0 expression microarrays (Illumina, USA) was obtained from NCBI Gene Expression Omnibus (GEO) database (GEO accession number: GSE13898; Kim et al., 2010). The 64 EAC patients were divided according to their expression pattern using an unsupervised hierarchical clustering analysis as previously described (Kim et al., 2010). Expression analysis was performed to compare the gene expression profile on the 64 EAC samples using the Agilent GeneSpring software v12.0 (Agilent Technologies). Significant differences in gene expression were determined by Student’s T-test. The p-values were further adjusted for multiple comparisons using the Benjamini-Hochberg FDR multiple testing correction, and was set at 0.05.

**Statistics**

$P$ value was calculated using chi-square in contingency table. Data are presented as mean ±
SD and were analyzed by 2-tailed Student’s t test. A P value of less than 0.05 was considered significant. Enhanced expression of NOTCH1 in EAC tumors versus normal mucosa was determined by the Mann-Whitney U Test. In all other cases, statistical significance was determined by Student’s T test. P value < 0.05 was considered statistically significant.
RESULTS

Elevated Notch activity is associated with the differentiation state and clinical stage of EAC, drives resistance to chemotherapy and results in poor prognosis.

In order to assess the status of the Notch pathway in EAC, we screened primary EAC samples for presence of NICD and expression of Notch target genes. NICD was present in 72.5% (29/40) of primary EAC tumor tissues via immunohistochemistry (IHC; Fig.1A). In contrast, only low levels of NICD can be detected in approximately 20% of cells in the basal layer of the normal esophageal mucosa (Fig.1A) and in cells of the normal gastric cardia (Fig.S1C). Similarly, western blot analysis of primary tumors and normal tissue displayed an increase in NICD expression (Fig.1B). The mRNA levels of Notch target genes (HES1, HEY1, HEY2, HEYL) and Notch ligands (JAG1, Jag2, DLL1, DLL3, DLL4) were also elevated in tumor samples compared to normal tissue (Fig.1C, Fig.S1A). We observed a similar increase in NICD and a commensurate increase in Notch target gene transcription in EAC cell lines as compared to normal cells (Fig.S2). When we compared the levels of NICD in EAC tumors relative to their stage and degree of differentiation we find that high levels of NICD were observed in 94.4% (17/18) of the poorly differentiated EAC tumor cases, whereas fewer and weaker positive nuclear staining for NICD was observed in 54.5%(12/22) of the well or moderately differentiated EAC cases ($P<0.0001$; Fig.1D,E). This indicates that Notch activity correlates with the stage of EAC (Fig.1F).

Current treatment guidelines for EAC include NAC followed by surgical resection. Patients that had a complete pathological response to NAC exhibited a significantly greater 5-year survival
compared to EAC patients that did not have a significant response. However, only 16% of patients experience a complete pathological response. The other 74% of EAC patients either had no response or partial response to NAC therapy (16-18). We sought to determine if Notch played a role in the response to NAC. Therefore, we analyzed a set of 28 surgically resected EAC samples derived from patients that failed NAC therapy for activation of the Notch pathway by IHC and q-PCR. Although Notch activity was elevated in the untreated group, the tumors that showed only a partial response, or were refractory to treatment, had appreciably greater levels of Notch signaling (Fig.2A,B,C). This indicates that tumors with elevated Notch activity may have been selectively enriched by chemotherapy or may be resistant to chemotherapy. To gain insight to this issue we analyzed the expression of NICD in chemo-naive EAC samples derived from ultrasound-assisted endoscopic (EUS) biopsies. Consistent with our hypothesis a patient sample that had undetectable levels of NICD had a complete response to chemotherapy, whereas two patients that had high levels of activated Notch did not show significant response to chemotherapy (Fig.2D). Therefore, activated Notch appears to predict response to chemotherapy. Consistent with these results an analysis of gene expression data from the Gene Expression Omnibus (GEO) indicates that activation of Notch signaling is associated with poor prognosis in EAC (Table 1).

**Notch activity promotes the proliferation and/or survival of EAC cell lines in vitro**

In order to assess the role of Notch signaling in cell proliferation and survival we blocked the Notch pathway by treating cells with DAPT, a commonly used GSI. Efficacy of the treatment was demonstrated by a dramatic reduction of NICD as seen by immunofluorescence in OE33
cells (Fig.3A). There was also a reduction in Notch-mediated transcription as seen by luciferase reporter activity and a decrease in transcription of Notch target genes (Fig.3B,C). Treatment of EAC cell lines with DAPT caused a decrease in cell viability (Fig.3D). The number and size of colonies formed by OE33 and JH-EsoAd1 cells treated with DAPT were significantly lower than those from mock treated cells suggesting that the reduction in proliferation is due to loss of Notch signaling. Inhibition of the Notch pathway did not alter the proliferation of Het-1A cells, a human immortalized esophageal epithelial cell line (Fig.3E bottom). To further validate this observation, and to rule out off-target effects of GSI, we knocked down the expression of CSL via shRNA (80-85% reduction; Fig.3F). There was a significant reduction in the proliferation and colony formation of OE33 cells with CSL knock down compared to control cells (Fig.3G, H). Together, these results suggest that the Notch pathway is required for proliferation and survival of EAC cells in vitro. Conversely, an increase in Notch signaling had the opposite effect. We established Het-1A/NICD and FLO1/NICD cell lines with stable expression of NICD. Ectopic activation of Notch pathway was confirmed by q-PCR and immunoblotting (Fig.S3). Het-1A/NICD and FLO1/NICD cells formed more colonies as compared to control cells harboring an empty vector (Fig.S3C, F), suggesting that increased activation of Notch signaling promoted the transformation of Het-1A cells and proliferation of FLO1 cells. Similar results were observed when exogenous NICD was expressed in OE33 cells (Fig.S3G). Collectively, this data indicates that NICD is a critical regulator of EAC cell proliferation and transformation of normal esophageal epithelial cells in vitro.

**Suppression of Notch activity inhibits tumor growth in EAC xenograft models**
To test the effect of Notch signaling on the growth of EAC cell lines xenografts, OE19 and OE33 cells were injected into the flank of immunocompromised mice. Once tumors reached 200 mm$^3$, we treated the animals with DAPT (20mg/kg) via daily intraperitoneal injections. Tumor growth was significantly stunted in the DAPT treatment group as compared to vehicle group for both cell line xenografts (Fig.4A, B). Compared to control, DAPT treatment reduced expression of the Notch target gene HES1 ($p=0.0119$; Fig.4C) and a decreased the level of NICD (Fig.4D). DAPT treatment also caused a decrease in proliferation and an increase in apoptosis as measured via Ki67 staining and TUNEL assay, respectively (Fig.4D). Furthermore, treatment completely inhibited the growth of OE19 cell xenografts when treatment was initiated one day following cell transplantation (Fig.4E). These data indicate that Notch activity is indispensable for tumor establishment and maintenance.

Since we observed that Notch activity was required for the initiation of xenograft tumor formation we wanted to determine if the residual tumor cells in a xenograft tumor treated with DAPT could reestablish a tumor. To address this point, OE19 xenograft tumors were established and treatment was initiated as previously described for 2 weeks. We observed that tumors treated with DAPT were significantly reduced in size compared to control group (Fig.4F). Following treatment, tumors were harvested and cell suspensions were prepared. Both mock and DAPT treated cell suspensions contained approximately 80% viable cells as determined by trypan blue exclusion. $1\times10^6$ viable cells from each treatment group were then transplanted into both flanks of 5 nude mice. Mock treated cells developed tumors while DAPT treated cells failed to develop any noticeable tumors 10 weeks after re-implantation (Fig.4F). The remaining
cells, we postulate, are comprised of bulk tumor cells that evidently lack the ability to reestablish a tumor in mice. Therefore inhibition of Notch signaling selectively abolished the subpopulation of cells capable of forming new tumors (i.e. tumor-initiating cells or CSCs) in the xenograft.

We sought to extend our findings using patient-derived xenografts (PDX). PDX models better represent the diversity of human cancer compared to cell line-based xenografts and are more representative of the original tumor. Therefore, the therapeutic efficacy in PDX models is a better predictor of the clinical response of the patient's tumor from which the model is derived. Three PDX models were established from EAC patients and these tumors display the same histological and IHC characteristics as the primary tumors (Fig.5A). Similar to cell line-based xenografts, inhibition of Notch signaling by DAPT in the PDX model lead to significantly stunted growth and a reduction in proliferation and increased apoptosis as compared to vehicle group (Fig.5B, C). Taken together, these data clearly demonstrate a critical role for Notch signaling in the proliferation and development of EAC tumors.

**EAC derived tumor spheres display characteristics of CSCs**

The experiment shown in Figure 4F indicates that inhibition of Notch signaling in the xenograft models is selectively targeting the CSC population of the tumor. Therefore, we sought to examine the role of Notch on the CSC population in EAC. To address this, a sphere-culture system was adopted for isolation/enrichment of EAC CSCs. Several publications have shown that cells grown under serum-free, low attachment conditions are enriched for specific stem-like characteristics (19). EAC cell spheres were obtained from cell lines after culture as
outlined in the methods (Fig.S4A). We were able to obtain cell spheres from all cell lines tested. A cell’s ability to form spheres was relative to the level of NICD present in the attached culture. OE33 cells have more NICD under attached conditions and are more efficient in forming spheres (Fig.6A, S4B). When compared to attached cells, all EAC cell line spheres had greater levels of NICD expression. To validate that EAC cell line sphere cultures were indeed enriched for cancer stem-cells, we analyzed a set of genes that mark stemness. All four cell lines have increased mRNA levels of several cell surface markers commonly used for the identification of cancer stem cell sub-population (ALDH1, CD133, CD25, LGR5, MSI2) as well as genes that mediate cellular de-differentiation (Twist, SNAIL, NANOG) and the maintenance of the stem cell phenotype (OCT4, MSI, SOX2, ZEB1; Fig.S4C). EAC cell spheres were also much more efficient in forming tumors. Injecting $5 \times 10^5$ OE33 sphere cells resulted in tumors of equal or greater size than those obtained by injecting $5 \times 10^6$ parental cells suggesting that the sphere cells are at least 10 times more efficient in forming xenograft tumors (Fig.S4D). Furthermore, as few as $5 \times 10^4$ sphere derived cells were able to initiate tumor formation in 5 weeks.

**CSCs marker genes are sensitive to inhibition of Notch signaling**

Once we established that EAC cell spheres formed xenografts more efficiently, we tested whether inhibition of Notch would inhibit growth of spheres. When compared to attached cells, spheres had higher transcription of Notch target genes (Fig. 6B). Treatment of OE33 spheres with DAPT reduced the amount of NICD and Notch target gene transcription (Fig.6B,C). GSI treatment also inhibits the ability of cell spheres to form secondary spheres (Fig.6D). In contrast, FLO1/NICD cells containing a constitutive active form of Notch had a greater sphere
forming potential as compared to FLO1/Control cells (Fig.6F).

To further confirm the role of Notch in the formation of EAC spheres, we treated sphere cultures with GSI and measured the expression level of several stem cell markers. The transcription of all stem cell marker genes assayed was higher in cell spheres versus attached cells. Inhibition of the Notch pathway caused a significant decrease in transcription ($p<0.001$) of several stem cell marker genes, including ALDH CD24, LGR5, SOX2 and TWIST1 (Fig.6E). This indicates that this set of CSC marker genes are directly regulated by Notch. The transcription of another subset of genes tested was increased in sphere conditions but not significantly altered by GSI treatment (SNAIL, MSI2, NANOG, OCT4, ZEB1); Indicating that the increase in transcription is due to the loss of stemness but independent of Notch signaling in EAC. GSI treatment of cell spheres increased the transcription of KLF4, which is in agreement with published data on the regulation of this gene by Notch. To validate this observation, we used FLO1/NICD cells and tested for the expression of two stem cell marker genes, SOX2 and OCT4. Exogenous expression of NICD increased the transcription of SOX2 seven fold compared to control cells but did not alter the transcription of OCT4, indicating that SOX2 is specifically regulated by Notch (Fig.6G). This was further confirmed by chromatin immunoprecipitation, which demonstrates that NOTCH1 binds to the SOX2 promoter in both FLO1 and OE33 cells (Fig.6H).

**EAC CSC population is more sensitive to inhibition of Notch than bulk tumor cells.**

Since EAC cell spheres show traits attributed to cancer stem cells, we sought to determine if their maintenance was dependent on Notch signaling. We tested adherent cells separated
according to their expression of ALDH, a common cancer stem cell marker (20), for their ability to grow in sphere media. Analysis of 4 EAC cell lines demonstrated that the ALDH positive cells displayed a 2-5 times greater ability to form spheres when compared to ALDH negative cells (Fig.7A). Additionally, treatment of attached cells with GSI decreased the ALDH⁺ fraction by 40%-60% (Fig.7B). Therefore, the EAC CSC population can be identified according to ALDH positivity and that this population of cells is more sensitive to inhibition of the Notch signaling than ALDH negative cells.

**Inhibition of Notch signaling sensitizes EAC for treatment with 5-FU**

The effect of blocking Notch on EAC CSCs has been characterized by various CSC assays. Notch signaling seems to promote the growth maintenance of tumor forming cells in EAC. There is also clear evidence that higher levels of Notch signaling in chemo naïve patients is a marker for poor prognosis following chemotherapy. In order to explore the role of Notch signaling on EAC chemoresistance, we performed a survival assay using 5-FU, a common agent used in EAC chemotherapy. We chose to use OE33 and FLO1 cells for this experiment because they have very high and low levels of NICD, respectively (Fig.6A). OE33 cells are insensitive to 5-FU treatment. Combination treatment of OE33 cells with GSI and 5-FU was more effective in killing OE33 (Fig.7C). In contrast, FLO1 cells are moderately sensitive to 5-FU. When NICD is ectopically introduced to FLO1 cells, these cells (FLO1/NICD) now exhibit a resistance to 5-FU (Fig.7D). Taken together these results suggest Notch signaling confers chemoresistance in EAC cells. Therefore, it is establishing proof-of-concept that combinatorial therapy with GSI and other chemotherapeutic agents will be a useful strategy in EAC.
treatment.
DISCUSSION

The incidence of esophageal adenocarcinoma (EAC) has shown a dramatic increase in the Western population (3,21). The 5-year survival rate of EAC is far below all other tumor types (5). Factors that have contributed to this poor 5-year survival are late diagnosis, resistance to chemotherapy and metastatic disease. (5,6). Current treatment regimens for EAC include neoadjuvant chemotherapy (NAC) followed by surgical resection. Various chemotherapy regimens have been used and typically include 5-fluorouracil, oxiplatin and docetaxel (17). In addition, radiation therapy can be given concomitantly with neoadjuvant chemotherapy. In spite of these treatment regimens the clinical response is relatively poor and this leads to dismal five year outcome. Local response rates to these therapies vary but for the vast majority, treatment results in only a modest downsizing or no response at all. Less than 20% of patients display a complete pathological response. However, it is these patients that obtain the greatest improvement in 5-year survival (17). Therefore, for improved clinical management of EAC, a treatment must improve the local response to neoadjuvant therapy. Herein we demonstrate that Notch signaling is critical for EAC and underlies resistance to chemotherapy. We present evidence that Notch signaling is greater in the less differentiated tumors and drives a cancer stem cell phenotype. We demonstrate that Notch signaling is critical for these EAC CSCs and that Notch regulates genes that establish stemness. Using patient derived xenograft models we clearly demonstrate that inhibition of Notch signaling by gamma-secretase inhibitors is efficacious in downsizing tumor growth. Moreover, we provide evidence that demonstrates Notch activity in a patient’s EUS-derived biopsy sample might predict outcome to
chemotherapy. Taken together, our data strongly suggest that Notch signaling drives a significant proportion of esophageal adenocarcinomas. Notch signaling does so by establishing and maintaining a cancer stem cell like population of cells, which also underlies resistance to chemotherapy. Therefore, inhibition of Notch depletes the CSC population and sensitizes cells to chemotherapeutic agents, which should lead to a better and more durable response to NAC. This study therefore provides a strong foundation for examining the efficacy of inhibitors of Notch signaling, such as GSI compounds, for the treatment esophageal adenocarcinoma. Given that Notch appears to drive CSCs and resistance to chemotherapy, one might expect the outcome of such clinical trials would be more profound on 5-year survival rate than in downsizing of the primary tumor itself as a single agent. Therefore, it would be a useful strategy to use GSIs to sensitize tumors to neoadjuvant chemotherapy and therefore improve both the local response and outcome of treatment. The use of a targeted therapy in combination with a typical chemotherapeutic and surgical treatment strategy will ultimately provide a more durable cure to this disease.
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REFERENCES


FIGURE LEGENDS

Figure 1. Notch activity elevated in esophageal adenocarcinoma (EAC), and associated with the differentiation state and clinical stage. A. NICD staining was shown in human EAC and normal esophageal mucosa tissues. B. Level of NICD was detected by Western blotting in EAC and adjacent normal mucosa. C. mRNA levels of Notch targets were determined by quantitative RT-PCR (q-PCR), and were normalized to the relative expression values of matched adjacent normal mucosa (set to 1). Error bars indicate ± SEM. D. Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining of NICD were determined in the EAC with well-, moderately-, and poorly differentiated tumors. E. Percent positive of NICD staining in the EAC with well/moderate- and poorly differentiated tumors (**P<0.0001). F. Percent positive of NICD staining in the EAC with different clinical stages (**P<0.0001). See also Fig. S1 and S2.

Figure 2. Elevated Notch activity drives resistance to chemotherapy in esophageal adenocarcinoma. A. Representative stainings of NICD were shown in the EAC without treatment, with partial response or resistant to chemotherapy. B. Percent positive of NICD staining in the groups (**P<0.0001). C. mRNA levels of HES1, NOTCH1 and NOTCH3 were determined by q-PCR, and were normalized to the expression values of GAPDH (set to 1). D. H&E and IHC staining of NICD in human esophageal ultrasound-assisted (EUS) biopsies.

Figure 3. Notch activity promotes the proliferation and/or survival of esophageal adenocarcinoma cell lines in vitro. A. Immunofluorescence (IFC) staining for NICD (red) in OE33 cells treated by DAPT for 3 days. B. Luciferase assays were performed in OE33 cells
previously infected with 8XCSL reporter and subsequently treated by DAPT for 3 days. Error bars indicate ± SEM. *P=0.0147, **P=0.0164, ***P=0.0004. C. mRNA levels of HES1 were determined by quantitative q-PCRs in OE33 cells treated by DAPT for 3 days. **P=0.0085, ***P=0.0022. D. Cell viability assays were performed in OE33 and OE19 cells treated by DAPT for 4 days in low serum medium (1% FBS). * P<0.05, ** P<0.01, *** P<0.001. E. Colony formation assays were performed in OE33, JH-EsoAd1 and Het-1A cells treated by DAPT for 7 days. F. CSL knockdowns were verified by Western blotting and q-PCRs in OE33 cells. *P=0.0016, **P=0.0020. G. Cell viability assays in OE33 cells infected with either control shRNA or shRNA against CSL. **P=0.0011, *P=0.0022. H. Colony formation assays in OE33 cells infected with shRNA against CSL and were quantified. **P=0.0025. See also Fig. S3

**Figure 4. Suppression of Notch activity inhibits tumor growth in xenograft models** A.B. DAPT (20mg/kg) daily treatment inhibited OE19 and OE33 cell line derived tumor growth in the mice xenograft models. *P<0.05, **P<0.01. C. mRNA levels of HES1 were determined by q-PCRs in OE19 derived tumors w/o DAPT treatment, and were normalized to the expression values of GAPDH (set to 1). P=0.0119. D. Representative images of OE19 derived xenografts treated by DAPT (lower panel) and vehicle (upper panel) with H&E and staining of NICD, Ki67 and TUNEL (green). E. DAPT treatment following cell transplantation inhibited the growth of OE19 cell xenografts. ***P<0.001. F. Tumor re-establishment from the residual tumor cells in the OE19 xenografts treated with DAPT (right) or vehicle (left) in nude mice. n=5, ***P<0.001.

**Figure 5. Suppression of Notch activity inhibits tumor growth in three EAC patient-derived xenograft models.** A. Same histological and IHC characteristics were
revealed in the primary tumors (up) and three patient-derived xenograft (PDX) models (down) established from EAC patients. B. DAPT (20mg/kg) daily treatment inhibited tumor growth of the three PDX models in NSG mice. n=6, Error bars indicate ± SEM, *P<0.05, **P<0.01. C. Representative images of NICD, Ki67 and TUNEL (green) staining in EAC28 derived PDX tumors treated by DAPT (down) and vehicle (up).

Figure 6. Cancer Stem Cell marker genes are sensitive to Notch signaling inhibition. A. Expressions of NICD in EAC adherent cells and spheres. B. mRNA levels of Notch targets were determined. The data were normalized to the values of control spheres. C. Expressions of NICD were detected by western blotting in OE33 spheres treated by DAPT for 3 days. C. Secondary sphere formation assays were done in OE33 cells after isolating from the first generation of spheres. The data were plotted by bar graph (up) and representative fields were shown (down). ***P<0.001. D. Tumor sphere formation assays done in FLO1/Control and FLO1/NICD cells. *P=0.0249. E. mRNA levels of stem cell transcription factors were determined in OE33 spheres treated by DAPT for 3 days. F. SOX2 and OCT4 mRNAs were detected by q-PCRs. ***P=0.0005. G. Notch1 on the SOX2 promoter was showed using chromatin immunoprecipitation (ChIP). See also Fig. S4

Figure 7. Notch inhibition selectively abolishes the CSC population, as well as sensitizes EAC for 5-FU. A. Tumor sphere assays were done in ALDH-sorted EAC cell lines. B. Flow cytometry analysis using ALDH in DAPT-pretreated EACs. C. Cell viability assays were performed in OE33 cells co-treated with 5-FU and DAPT for 4 days. *P<0.05, **P<0.01, ***P<0.001. D. Cell viability assay in FLO1/Control and FLO1/NICD cells treated by 5-FU for 4
days. **$P=0.0023$, ***$P=0.0006$. 
Figure 1

A

Normal

Tumor

NICTD

EAC04 EAC16 EAC24 EAC25 EAC27 EAC28

N  T  N  T  N  T  N  T  N  T  N  T

GAPDH

B

NICTD

GAPDH

C

Relative Gene Expression

(EAC04 EAC16 EAC24 EAC25 EAC27 EAC28)

D

Well Differentiated

Moderately Differentiated

Poorly Differentiated

E

Percent Positive

Well / Moderately Poorly

Differentiation

F

Percent Positive

Clinical Stage

I II III/IV

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Figure 2

A

Not Treated

Partial Response

No Response

B

Percent Positive

Not Treated
Partial Response
Resistant

C

HES1 mRNA

Notch1 mRNA

Notch3 mRNA

D

Chemo Naïve Samples from EUS

EAC-EUS01
EAC-EUS02
EAC-EUS04

H&E

NICD

Response to Chemo

Complete Response
No/Partial Response
**Figure 3**

**A**

Immunofluorescence images showing DAPI (blue), NOTCH1 (red), and DAPI/NOTCH1 (merged) channels in the presence of DMSO or DAPT.

**B**

Bar graph showing luciferase activity of 8xCSL in EAC cells treated with DMSO or DAPT at 1, 5, and 10 μM. DAPT treatment significantly decreases luciferase activity compared to DMSO.

**C**

Bar graph showing relative gene expression of HES1 mRNA in EAC cells treated with DMSO or DAPT at 1 and 5 μM. DAPT treatment significantly decreases HES1 mRNA expression compared to DMSO.

**D**

Bar graphs showing cell viability of OE33 and OE19 cells treated with DMSO or DAPT at 1, 5, and 10 μM. DAPT treatment significantly decreases cell viability compared to DMSO.

**E**

Images of EAC cells treated with DMSO (1 μM and 5 μM) and DAPT at 5 μM, showing a decrease in cell viability with DAPT treatment.

**F**

Bar graphs showing relative gene expression of CSL in OE33 cells treated with shControl or shCSL#2 and shCSL#5. CSL expression is significantly reduced in shCSL#2 and shCSL#5 compared to shControl.

**G**

Bar graphs showing cell viability of Het-1A cells treated with shControl or shCSL#2 and shCSL#5. CSL knockdown significantly decreases cell viability compared to shControl.

**H**

Bar graph showing the number of colonies in EAC cells treated with shControl or shCSL#2 and shCSL#5. CSL knockdown significantly decreases the number of colonies compared to shControl.
Figure 6

Panel A: Western blot images showing NICD and GAPDH levels in OE33, OE19, FLO1, and JH-EsoAd1 cell lines under control and DAPT treatment conditions. Levels are quantified as P and S.

Panel B: Bar chart showing relative expression of Notch1, Notch3, HES1, and HEYL for Parental Cells, Sphere+DMSO, Sphere+DAPT1uM, and Sphere+DAPT5uM conditions.

Panel C: Western blot images showing NICD and GAPDH levels under Control and DAPT treatment conditions with various concentrations (1uM, 5uM, 10uM).

Panel D: Graph showing number of spheres formed under DMSO and DAPT treatment conditions. Numbers are marked as Control, 1uM, 5uM, and 10uM.

Panel E: Bar chart showing relative expression of various genes (ALDH1A1, CD133, CD24, LGR5, SOX2, TWIST1, SNAI1, MS12, NANO2, OCT4, ZEB1, KLFA) under Parental Cells, Sphere+DMSO, and Sphere+DAPT5uM conditions.

Panel F: Graph showing spheres number (1000 cells) under FLO1/Control and FLO1/NICD conditions.

Panel G: Graph showing relative gene expression under FLO1/Control and FLO1/NICD conditions.

Panel H: Diagram illustrating the region of interest (TTCCCA) in the Human SOX2 gene with TSS marked and Exons 1 and 2 labeled.
Figure 7

A

Presort

DEAB

Control

ALDH Negative

ALDH Positive

Spheres Number (1000 cells)

FLO1  JH-EsoAd1  OE33  OE19

B

Control

DAPT

Percent ALDH Positive

FLO1  JH-EsoAd1  OE33  OE19

C

Relative Cell Viability (%)

OE33

Relative Cell Viability (%)

OE33

D

FLO1

DMSO  1  5  10

5-FU (μM)

Relative Cell Viability (%)

FLO1/Control  FLO1/5ICD
Notch Signaling Drives Stemness and Tumorigenicity of Esophageal Adenocarcinoma


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